The evolution of androgen receptor expression and behavior in *Anolis* lizard forelimb muscles

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13 **Abstract**

14 The motor systems that produce behavioral movements are among the primary targets for the action of

15 steroid hormones, including androgens. Androgens such as testosterone bind to androgen receptors (AR)

16 to induce physiological changes in the size, strength, and energetic capacity of skeletal muscles, which

17 can directly influence the performance of behaviors in which those muscles are used. Because tissues

18 differentially express AR, resulting in tissue-specific sensitivity to androgens, AR expression may be a

19 major target of selection for the evolution of behavior. *Anolis* lizards (i.e., anoles) provide a robust system

20 for the study of androgen-regulated traits, including the behavioral traits that facilitate social display and

21 locomotion. In this study, we examined six anole species that demonstrate significant variation in the

22 behavioral use of the forelimbs to measure the proportion of myonuclei in the bicep muscles that express

23 AR. Using phylogenetic comparative analyses, we found that species with a greater proportion of nuclei

24 positive for AR expression in the biceps exhibited greater frequencies of locomotor movements and

25 pushup displays. These results suggest that AR expression in skeletal muscles may influence the

26 evolution of androgen-regulated behaviors in this group.

27

28 **Keywords**: androgen receptor; *Anolis* lizards; bicep; pushup; locomotion

29

Introduction

An animal's performance of a complex behavior requires the integration and transmission of signals from the central nervous system to the peripheral motor systems, which ultimately produce the behavioral movements. Because even closely-related species may vary extensively in movement patterns (e.g., Prum 1990; Hale et al. 2002; Ord and Martins 2006), studies of the physiological differences in musculoskeletal systems may provide critical insights into the evolution of this behavioral variation. While a robust body of work has explored the relationships between motor systems and behavior within individual model species, phylogenetic comparative studies that explicitly examine the evolution of the endocrine mechanisms of behavior remain somewhat rare (but see e.g., Hale et al. 2002; Johnson and Wade 2010; Fuxjager et al. 2015; Mangiamele et al. 2016).

The action of steroid hormones, including androgens, is one of the primary physiological bases of vertebrate social behavior and performance. Androgens play significant roles in the development, maintenance, and expression of male reproductive morphology and behavior (reviewed in Adkins-Regan 2005), and across vertebrates, the peripheral motor system is a major androgenic target (e.g., Rand and Breedlove 1992; Brantley et al. 1993; Regnier and Herrera 1993; Veney and Wade 2004). In particular, androgens influence the size, strength, and growth rates of skeletal muscles (Herbst and Bhasin 2004), often in sex- or species-specific patterns (e.g., Cox et al. 2009), yet they can also affect cellular processes such as respiration and metabolism within muscle cells. In the only study to date on androgenic effects on the muscular transcriptome, Fuxjager et al. (2016) found that in the forelimb musculature of two passerine bird species (golden-collared manakins, *Manacus vitellinus*, and zebra finches, *Taenopygia guttata*), testosterone differentially regulated gene expression in species- and muscle-specific patterns. Most notably, androgens influenced expression in genes and gene networks associated with the contractile force and energetic capacity of the muscle fibers, traits with wide-ranging potential effects on an animal's behavioral use of a muscle.

The presence of these tissue-specific androgenic effects suggests that a given level of circulating plasma androgens can differentially affect tissues within the same organism. The most likely mechanism

56   for such an effect is the differential expression of androgen receptors (AR), as tissues with greater AR

57   expression are likely more sensitive to the action of androgens than tissues with lower AR. This offers a

58   clear target of selection for the evolution of behavior: variation in the use of skeletal muscles across

59   species may be controlled by variation in AR expression in those muscles. In comparative studies of

60   manakins (Fuxjager et al. 2015) and frogs (Mangiamele et al. 2016), this hypothesis received strong

61   support: species in which limb musculature is used to perform elaborate behavioral movements exhibit

62   greater AR expression in those muscles than species that do not perform these movements, and they

63   exhibit greater AR expression in muscles used to perform these movements than in muscles not

64   associated with the behaviors. Yet, these studies focus on complex behaviors that are unique to the taxa of

65   study: wing-snap sonations in golden-collared manakins and foot-flagging displays in Borean rock frogs

66   (*Staurois parvus*). However, differential AR expression may also drive the evolution of muscle use in

67   highly frequent behaviors such as locomotion, or simple social displays such as lizard pushups.

68   Lizards in the genus *Anolis* (i.e., anoles) provide an excellent system for studies of the evolution

69   of the physiological mechanisms underlying behavior. Anoles are extraordinarily diverse, with

70   approximately 400 species that occur throughout the Caribbean, Central and South America, and the

71   southeastern United States (Losos 2009). There is a long history of field and laboratory studies of this

72   group, providing extensive information on the diversity of their behavior, ecology, and morphology

73   (reviewed in Losos 2009), and a robust phylogeny is available for the genus (Pyron et al. 2013). Further,

74   the neural, muscular, and endocrine mechanisms of social behaviors have been well studied in one anole

75   species, *Anolis carolinensis* (the Carolina green anole; reviewed in Wade 2012), including quantification

76   of AR in myonuclei (Holmes and Wade 2005, Neal and Wade 2007).

77   Among the most common behaviors exhibited by anoles are locomotor movements and social

78   displays, behaviors that are easily observed in the field and readily quantified. Anoles primarily use

79   arboreal perches such as tree trunks and branches, bushes, or tall grass, and they move along and among

80   perches using crawls, runs, and jumps (e.g., Irschick and Losos 1998). In the Caribbean, most anole

81   species exhibit specializations to particular microhabitats (e.g., tree trunks, or grasses and bushes), and

82   these microhabitat specialists (called ecomorphs) vary in their rates of locomotion in these habitats

83   (Johnson et al. 2008). Social displays in this group generally consist of species-specific combinations of

84   pushups and head-bobs, performed in conjunction with the extension of a throat fan called a dewlap, and

85   in some species, with display modifiers such as the development of an eyespot or raising a nuchal crest

86   (Jenssen 1977). These displays are performed by both sexes during courtship, territorial defense, and

87   predator pursuit deterrence (Leal and Rodriguez Robles 1997), or in the context of species recognition

88   (Nicholson et al. 2007), with males displaying far more frequently than females (e.g., Jenssen et al. 2000).

89   Although levels of circulating androgens vary among anole species, these differences are not associated

90   with variation in aggressive display behaviors (Husak and Lovern 2014). This suggests that instead, AR

91   expression in the skeletal muscles that control display behaviors may mediate the interaction between

92   androgens and behavior in this group.

93   Both pushup displays and locomotor behaviors require the contraction of a suite of forelimb

94   skeletal muscles. First, biceps have been hypothesized (Herrel et al. 2008, Anzai et al. 2014) and

95   demonstrated (Foster and Higham 2014) to be important in elbow flexion (decrease in the angle between

96   the forearm and the hind limb) during locomotion, a movement also involved in the pushup display. In

97   humans, the biceps are a major contributor during pushup behavior, and variation in bicep activation is

98   correlated with pushup speed, such that slower pushups result from greater bicep activation (Chou et al.

99   2011). Other major contributors to the pushup movement in humans are the supinator, pronator teres,

100   triceps brachii, middle deltoid, anterior deltoid, pectoralis major and posterior deltoid (Chou et al. 2011).

101   All of these muscles are present in lizards (Wheeler 1939, Suzuki et al. 1996) and some have been

102   specifically documented in *Anolis* lizards (Anzai et al. 2014). In particular, Anzai et al. (2014) report that

103   the anterior deltoid (M. clavodeltoideus) functions as a humeral protractor, the posterior deltoid (M.

104   scapulodeltoideus) functions as a humeral abductor, the pectoralis major (M. pectoralis profundus)

105   functions as a humeral adductor, and the triceps brachii (of the triceps complex) function as an elbow

106   extensor in *Anolis*. Humeral abduction, adduction, and protraction, and elbow extension are all

107   movements that occur during locomotion and pushup displays.

108   In this study, we tested the hypothesis that AR expression in anole forelimb muscles, and in the

109   biceps in particular, is positively associated with the behavioral use of the forelimbs. To this end, we

110   selected six *Anolis* species that represent a wide range of locomotor movement and display rates (Johnson

111   et al. 2008, Johnson and Wade 2010). These six species include *A. carolinensis* from the southeastern

112   United States, and five species from southwestern Dominican Republic: *A. bahorucoensis* (the Bahoruco

113   long-snouted anole), *A. brevirostris* (the shortnose anole), *A. coelestinus* (the Hispaniolan green anole), *A.*

114   *cybotes* (the largehead anole), and *A. olssoni* (the desert grass anole; Figure 1). In this species group, we

115   predict that species that perform higher rates of locomotor movements and pushups will express more AR

116   in biceps than those that perform these behaviors less frequently.

117

118   **Materials and Methods**

119   *Field Behavioral Data and Tissue Collection*

120   To quantify differences in locomotor and pushup behaviors, we conducted standardized

121   behavioral observations on adult male lizards of each of six *Anolis* species (Table 1) during five summer

122   breeding seasons across 2006-2015, as follows. Observations occurred between 0700 and 1800, and never

123   during inclement weather (i.e., rain), as lizards may take refuge during those times (Hertz et al. 1993).

124   Individuals were located for observations by walking slowly through the field sites, and undisturbed

125   lizards were observed for 10-120 min from a minimum distance of 10 m. In 2006, 2010, and 2014

126   observations, anoles within a study plot were individually marked using bead tags sewn into the tail

127   muscle (Fisher and Muth 1989), and released at their site of capture. After a minimum of 24 h post-

128   capture, we conducted undisturbed focal observations of marked individuals (up to a maximum of 3 h per

129   individual), and calculated average rates of pushup displays and locomotor movements for each

130   individual (see Johnson et al. 2010 and Bush et al. 2016 for details). In 2011 and 2015 observations, we

131   observed unmarked males. As male anoles are generally thought to remain in a small home range or

132   territory (Stamps 1977, 1994, but see Kamath and Losos 2017), we avoided repeated observations in the

133   same immediate area to minimize the probability of performing multiple observations on the same lizards.

134 During observations, we recorded all locomotor behaviors (with each movement defined as a crawl, run,

135 or jump) and pushup displays. We calculated the average rates of locomotor movements and pushup

136 displays for each species for use in statistical analysis. Some data for these and additional behavioral traits

137 collected during these observations were previously reported in Johnson et al. (2008; foraging mode),

138 Johnson et al. (2010; territorial defense), Johnson and Wade (2010; dewlap display), Dill et al. (2013;

139 locomotor behavior in *A. carolinensis*), Johnson et al. (2014; copulation), and Bush et al. (2016; territorial

140 behavior of *A. carolinensis*).

141   In 2011, we captured *A. brevirostris*, *A. coelestinus*, *A. cybotes*, and *A. olssoni* for measures of

142 bicep muscle tissues by hand at night on July 11, 2011, on the grounds surrounding Coralsol Beach

143 Resort in Barahona, Dominican Republic; and *A. bahorucoensis* on July 11, 2011 in the montane region

144 near Polo, Dominican Republic. We collected *A. carolinensis* in in mid-July 2011 at Palmetto State Park

145 in Gonzales, Texas. Each of these localities were the same localities in which behavioral observations of

146 the species occurred, but the individuals for which behavioral data were collected were different

147 individuals than those used for tissue analysis. We kept lizards in air-filled plastic bags upon capture and

148 moved them to cloth bags for transport. Upon arrival at Trinity University, at most two days after capture,

149 we measured each lizard's snout-vent length (SVL) using Mitutoyo digital calipers (to the nearest 0.5

150 mm), and mass (to the nearest 0.1 g) using Pesola spring scales. The lizards were then immediately

151 euthanized via rapid decapitation, and muscles from the forelimb (including the biceps) were flash-frozen

152 on dry ice and stored at -80°C until further processing.

153

154 *Quantification of AR in Muscle Tissues*

155   We sectioned frozen forelimb muscle tissues with a cryostat at 20 μm in 6 series (i.e., multiple

156 sections were collected on a single slide at 120 μm intervals) and thaw-mounted the tissues on SuperFrost

157 Plus microscope slides (Fisher Scientific; Hampton, NH, USA). We sectioned the medial portion of each

158 forelimb on a transverse plane, such that cross-sections of the muscle tissues were examined. Sectioned

159 tissues were stored at -80 °C until we performed immunocytochemistry.

160   To measure AR in the bicep tissues, we adapted immunocytochemistry protocols previously used

161   in green anole lizard (*Anolis carolinensis*) muscle tissues, as described in Holmes and Wade (2005) and

162   Neal and Wade (2007). All tissues were processed in a single immunocytochemistry run. In brief, slides

163   from a single series were warmed to room temperature, and tissues were fixed in 4% paraformaldehyde in

164   0.1 M phosphate buffered saline (PBS) for 10 min. Slides were rinsed 3 times for 5 min in 0.1M PBS

165   between all steps. Following a 30 min incubation in 0.5% H_2O_2 to remove endogenous peroxidases, slides

166   were incubated in 4% normal donkey serum in 0.1 M PBS with 0.2% Triton X-100 for 2 h, and then in

167   PG21 rabbit polyclonal antibody (1.75 ug/ml; EMD Millipore, Billerica, MA, USA) in 0.1 M PBS with

168   0.2% Triton X-100 and 30% glycerol, for 48 h at 4°C. After incubation with primary antibody, slides

169   were rinsed three times in PBS and then incubated in biotinylated donkey antirabbit secondary antibody

170   (Jackson Laboratories, West Grove, PA, USA) at a dilution of 1:500 in 0.1M PBS, for 90 min. Slides

171   were then incubated in Elite ABC peroxidase reagents (Vector Laboratories, Burlingame, CA, USA) for

172   60 min to visualize AR immunoreactive (AR+) myonuclei. We then stained tissues for 5 min with 4', 6-

173   diamidino-2-phenylindole (DAPI; 1 ug/ml; Sigma, St Louis, MO, USA) to visualize all myonuclei.

174   Finally, tissues were dehydrated in a series of ethanols, defatted in xylene, and coverslipped with DPX

175   mounting medium.

176   To confirm the specificity of the antibodies used, we used two negative controls. First, the

177   primary antibody was preadsorbed with 20x molar mass of the AR protein (custom peptide of the first 21

178   amino acids of the human AR protein, the peptide against which PG-21 was raised, Biosynthesis, Inc.,

179   Lewisville, TX). In addition, we ran a no-primary control for each species, in which tissue sections were

180   exposed to the same ICC protocol, but with the primary antibody omitted. Nuclei labelling was minimal

181   in all sections exposed to these control protocols.

182   For each lizard, we determined the number of AR+ nuclei in the biceps in a 320 μm x 320 μm

183   area near the rostrocaudal center of the biceps, on each side of the animal. We then counted the total

184   number of myonuclei in the same area, counting only nuclei within the muscle fibers. Following Holmes

185 and Wade (2005) and Neal and Wade (2007), we then calculated the proportion of AR+ nuclei from the

186 total myonuclei for each individual.

187 Finally, we also measured the cross-sectional area of the biceps muscle fibers for each individual

188 of all species (except *A. bahorucoensis*, for which alternate series of sectioned tissue were not available).

189 We stained an alternate series of tissues with hematoxylin and eosin, and using ImageJ, measured the size

190 of 20 arbitrarily selected fibers on each side of the animal, in the medial portion of the rostrocaudal center

191 of the muscles. These measures were then averaged for each individual.

192

193 *Statistical Analysis*

194 Because the data in this study were non-normally distributed, we transformed each behavioral

195 variable by taking the logarithm of the raw value + 1, and we performed an arcsine-square root

196 transformation for the proportion of AR+ nuclei in the biceps. We used transformed data in all subsequent

197 analyses.

198 To determine if the six species differed in rates of pushup display and locomotor movements, we

199 used a series of Analyses of Variance (ANOVA), followed by Tukey's HSD post hoc tests. To determine

200 the evolutionary relationships between AR expression, behavior, and morphology, we performed

201 phylogenetic analyses using the squamate phylogeny in Pyron et al. (2013), pruned to include only the

202 species in this study. We conducted a regression using a phylogenetic generalized least squares (PGLS)

203 regression analysis, using the caper package (Freckleton et al. 2002) in R (R Core Team 2014), to

204 determine whether the proportion of AR+ nuclei in the biceps was associated with rates of pushups or

205 locomotor movements. To determine whether rates of pushups and locomotion were correlated, and

206 whether the proportion of AR+ nuclei was correlated with muscle fiber size or body mass across species,

207 we used generalized least squares correlation assuming Brownian evolution of traits, using the *gls*

208 function in the nlme package (Pinheiro et al. 2017) in R.

209

210 **Results**

211   The six species in this study differed dramatically in their use of the forelimbs during behavioral

212 observations (Table 2). The rates of pushup displays ranged from those who rarely exhibit this behavior

213 (*A. bahorucoensis*) to those that perform pushups more than 8 times per min (*A. carolinensis*), with the

214 other four species exhibiting intermediate pushup rates (ANOVA: $F_{5,410} = 68.4$, $p < 0.001$). Similarly, the

215 rate of total locomotor movements range broadly ($F_{5,386} = 58.1$, $p < 0.001$), with the same two species

216 exhibiting the most extreme behaviors; *A. carolinensis* moved 9x more frequently than *A. bahorucoensis*

217 (Table 2). Among the other species, post-hoc tests revealed that *A. brevirostris* and *A. coelestinus* moved

218 at intermediate rates, and *A. olssoni* and *A. cybotes* moved relatively rarely (Table 2). We performed all

219 analyses with both the rate of total locomotor movements, and the rates of the three types of movement

220 (runs, crawls, and jumps) considered separately. Because the results were qualitatively similar in these

221 analyses, we only present subsequent analyses with total movement rates below.

222   The average proportion of AR+ nuclei in the biceps ranged across the six species from 0.255 (*A.*

223 *cybotes*) to 0.385 (*A. carolinensis*), although these means did not statistically differ (ANOVA: $F_{1,5} = 1.41$,

224 $p = 0.246$). The effect size for this analysis is fairly low (partial $\eta^2 = 0.18$), suggesting that the small

225 sample size included here may not be sufficient for detecting statistical significance. Further, there was

226 substantial variation within several species, with the most extreme value belonging to an *A. carolinensis*

227 individual whose proportion of AR+ nuclei was 0.16, less than half the average value for the species

228 (although this was not a statistical outlier and so was not removed from the analysis).

229   Across the six species, the behavioral use of the forelimbs increased in association with the

230 average proportion of AR+ nuclei in the biceps. The rate of locomotor movements was positively

231 associated with AR expression in the biceps muscles (PGLS: Adj. $R^2 = 0.77$, $F_{1,4} = 17.29$, $p = 0.014$,

232 Figure 2a), and the rate of pushup displays was positively associated with biceps AR (PGLS: Adj. $R^2 =$

233 0.59, $F_{1,4} = 8.29$, $p = 0.045$, Figure 2b). In addition, the rates of locomotion and pushup displays were

234 highly correlated with one another across these six species (phylogenetic correlation: $p = 0.018$).

235   The variation across species in AR+ nuclei was not associated with the mass of the animals

236 (phylogenetic correlation: $p = 0.89$) or the size of the fibers in the biceps (phylogenetic correlation: $p =$

237 0.075). Pushup rate and locomotor rate were also not associated with bicep fiber size (phylogenetic

238 correlation, pushups: p = 0.37, locomotion: 0.24), or body size (phylogenetic correlation, pushups: p =

239 0.25, locomotion, p = 0.59).

240

Discussion

242   The results of this study support the hypothesis that the evolution of AR expression in skeletal

243 muscles is associated with the evolution of the behavioral use of those muscles (Fuxjager et al. 2015;

244 Mangiamele et al. 2016). Although this relationship appears to be strongly influenced by the green anole,

245 *A. carolinensis*, which exhibits the highest rates of locomotor and display behaviors and has the highest

246 average AR expression in the biceps (Figure 2), the direction of the relationship is as we predicted.

247 Together, these results suggest that the overall behavioral use of the biceps (and not just the muscle's use

248 in a particular behavioral context) is associated with the concentration of receptors in its muscle fibers.

249 For example, highly active lizards that aggressively defend a large territory from potential intruders

250 would require muscles that could support both frequent movements around the territory and repeated

251 displays to the intruders. Alternatively, increased AR expression in the biceps may have been selected to

252 facilitate the use of the muscles in a specific context (such as in locomotion), and the receptors are then

253 available to support the muscle's use in other contexts (such as social display). Further, there may be

254 other uses of the forelimb driving the evolution of AR expression that were not considered in this study.

255 For example, male frogs frequently use their forelimbs to grasp females during mating (Hannigan and

256 Kelley 1986, Peters and Aulner 2000). While the use of forelimbs does not appear to play a critical role in

257 anole mating behavior, we did not directly examine it here.

258   In our selection of species for this study, we aimed to include species that represented the full

259 range of reported anole movement and display rates (Johnson et al. 2008, Johnson and Wade 2010).

260 Although the observation time varied across the six species in this study, the patterns of behavior reported

261 here are highly consistent with previous descriptions of the behavior of these species (e.g., Jenssen and

262 Gladson 1984, Queral et al. 1995, Jenssen and Nunez 1998, Orrell and Jenssen 1998). Our selection of

263 species also resulted in a group in which locomotion and display rates were positively correlated, such

264 that we cannot decouple the effects of AR expression on the two behaviors. Yet, locomotion and display

265 rates are not necessarily coupled across all anole species. For example, species that primarily occur on

266 small twigs in the canopy (i.e., species in the "twig" ecomorph) generally move frequently, actively

267 foraging among the foliage, but display rarely (Hicks and Trivers 1983; Huyghe et al. 2007). On the other

268 hand, species that primarily perch low on tree trunks with their heads oriented toward the ground (i.e.,

269 species in the "trunk-ground" ecomorph), are generally sit-and-wait predators that move rarely, but

270 display frequently from their highly visible perches (Rand 1967; Losos 1990). This study included only

271 one trunk-ground species (*A. cybotes*), and no twig species. Thus, future work could examine AR

272 expression in a broader diversity of species to determine whether AR expression in the forelimb

273 musculature is more closely associated with either locomotor behavior or display, or if the total use of the

274 muscles, regardless of behavioral context, correlates with AR expression.

275 Increased AR expression may have many effects on skeletal muscle and the behaviors the muscle

276 supports. Higher AR may allow greater utilization of circulating androgens, potentially altering the

277 physiological capacity of the muscle. Using RNA-Seq, Fuxjager et al. (2016) found a wide variety of

278 androgen-regulated genes that likely influence muscle movement in two passerine bird species. These

279 genes and gene networks primarily included those that control lipid and carbohydrate metabolism and

280 mitochondrial function, although androgens also influenced basic cellular functions such as ion and

281 protein transport. Fuxjager and colleagues (2016) concluded that androgenic effects on skeletal muscles

282 can increase fiber contractile strength and/or the energy-producing capacity that supports the endurance

283 needed for muscular activity, although these effects may be species- or muscle-specific. In support of

284 these transcriptional effects, experimental exposure to androgens also alters muscle fiber types in species-

285 and muscle-specific patterns (e.g., Rubinstein et al. 1983, Eason et al. 2000, Holmes et al. 2007),

286 demonstrating tissue-level changes in muscular metabolic capacity and strength.

287 Variation in AR expression can also interact with variation in circulating androgen levels to

288 influence androgen-sensitive phenotypes. In anole lizards, as in many other vertebrates, the direct

289 manipulation of testosterone alters a range of male phenotypes, including aggressive behaviors and social

290 displays (e.g., Tokarz et al. 2002; Neal and Wade 2007; Cox et al. 2009). Husak and Lovern (2014)

291 reported extensive variation in circulating testosterone among 18 *Anolis* species on four Caribbean islands.

292 Surprisingly, they found a negative relationship between species aggression and testosterone in three of

293 the four assemblages, including several species from the Dominican Republic examined here, although

294 they found no relationship between testosterone and rates of social display across the group (Husak and

295 Lovern 2014). Among the six species in the current study, measures of circulating testosterone are

296 available for four: *A. brevirostris*, *A. coelestinus*, and *A. cybotes* (Husak and Lovern 2014) and *A.*

297 *carolinensis* (Husak et al. 2007). Although there are only four species, there is a marginally significant

298 relationship between these previously reported testosterone measures and the levels of AR expression in

299 biceps reported here (Figure 3; phylogenetic correlation: p = 0.051). Similarly, Holmes and Wade (2005)

300 found that within the green anole, *A. carolinensis,* testosterone increases the number of AR+ myonuclei in

301 a muscle involved in copulation (the retractor penis magnus, which controls movement of the hemipenes),

302 but not in the muscle that controls movement of the dewlap, the throat fan used during social display (the

303 ceratohyoid). Together, these results tentatively suggest that testosterone may differentially upregulate

304 the expression of its receptors in the anole peripheral motor systems.

305   Finally, intraspecific variation in muscular AR and behavior has also been examined within the

306 green anole. Neal and Wade (2007) measured AR expression in the ceratohyoid and the retractor penis

307 magnus in unmanipulated adult males. In contrast to the interspecific relationship between biceps AR

308 expression and behavioral use reported here, Neal and Wade (2007) found that variation in muscle AR

309 was not associated with individual behavioral variation in male courtship or copulatory behaviors, and

310 suggested that muscular tissues varied in androgen sensitivity by a mechanism other than variation in AR

311 expression. This suggests that different endocrine mechanisms may regulate behavioral variation within a

312 species than those driving the evolution of behavioral variation among closely-related species. While

313 intraspecific mechanisms may be conserved across evolutionary time, simply modified to give rise to

314 behavioral diversity among species, interspecific changes in behavior could arise due to changes in

315 pathways other than those used to create or maintain intraspecific behavioral variation.

316   In sum, we report an evolutionary correlation between behavioral phenotype and hormone

317 receptor expression. Though causative studies on AR's influence on behavior have not yet been

318 performed, correlative evidence for hormone receptor concentration causing behavioral variation is

319 mounting, and there is no evidence that the behavioral use of a muscle mechanistically influences its

320 hormone receptors. Indeed, Fuxjager and colleagues (2015) argued that, in manakins, behavioral

321 differences among species could not account for variation in AR expression, because in species in which

322 males (who use forelimb musculature in display) have high forelimb AR, females (who do not display)

323 also have high AR. Instead, variation in receptor expression likely causes tissue-specific responses to

324 globally circulating hormones that are often secreted by organs that are long distances from the tissues on

325 which they act. This hypothesis has been supported in studies of the effects of hormone receptors on

326 skeletal morphology. For example, Zheng and Cohn (2011) demonstrated that differences in AR and

327 estrogen receptor densities between the fourth and second digits of developing mouse hands influence

328 sexual dimorphism in digit length ratios, and Sanger et al. (2014) found that changes in the concentration

329 of estrogen receptors determined sexually dimorphic face length in anole lizards. Together with our

330 results, these data show the potential for selection to alter phenotypes that are regulated by hormones

331 through the evolution of hormone receptors in the peripheral musculoskeletal systems.

332

344 **References**

345 Adkins-Regan E (2005) Hormones and Animal Social Behavior. Princeton University Press, Princeton

346 Anzai W, Omura A, Diaz AC, Kawata M, Endo H (2014) Functional morphology and comparative

347   anatomy of appendicular musculature in Cuban *Anolis* lizards with different locomotor habits.

348   Zool Sci, 31:454-463. doi: 10.2108/zs130062

349 Brantley RK, Marchaterre MA, Bass AH (1993) Androgen effects on vocal muscle structure in a teleost

350   fish with intersexual and intrasexual dimorphism. J Morphol 216:305-318

351 Bush JM, Quinn MM, Balreira EC, Johnson MA (2016) How do lizards determine dominance? Applying

352   ranking algorithms to animal social behavior. Anim Behav 118:65-74. doi:

353   10.1016/j.anbehav.2016.04.026

354 Chou PPH, Hsu HH, Chen SK, Yang SK, Kuo CM, Chou YL (2011) Effect of push-up speed on elbow

355   joint loading. J Med Biol Eng 31:161-168. doi: 10.5405/jmbe.772

356 Cox RM, Stenquist DS, Calsbeek R (2009) Testosterone, growth, and the evolution of sexual size

357   dimorphism. J Evol Biol 22:1586-1598. doi: 10.1111/j.1420-9101.2009.01772.x

358 Dill AK, Sanger TJ, Battles AC, Johnson MA (2013). Sexual dimorphisms in habitat-specific morphology

359   and behavior in the green anole lizard. J Zool 290:135-142. doi: 10.1111/jzo.12020

360 Eason JM, Schwartz GA, Pavlath GK, English AW (2000) Sexually dimorphic expression of myosin

361   heavy chains in the adult mouse masseter. J Appl Physiol 89:251-258

362 Fisher M, Muth A (1989) A technique for permanently marking lizards. Herpetol Rev 20(2):45-46.

363 Foster KL, Higham TE (2014) Context-dependent changes in motor control and kinematics during

364   locomotion: modulation and decoupling. Proc Roy Soc B: Biol Sci 281:20133331. doi:

365   10.1098/rspb.2013.3331

366 Freckleton RP, Harvey PH, Pagel M (2002) Phylogenetic analysis and comparative data: a test and review

367   of evidence. Am Nat 160:712-726. doi: 10.1086/343873

368 Fuxjager MJ, Eaton J, Lindsay WR, Salwiczek LH, Rensel MA, Barske J, Sorenson L, Day LB, Schlinger

369   BA (2015) Evolutionary patterns of adaptive acrobatics and physical performance predict

370   expression profiles of androgen receptor – but not oestrogen receptor – in the forelimb

371   musculature. Funct Ecol 29:1197-1208. doi: 10.1111/1365-2435.12438

372   Fuxjager MJ, Lee JH, Chan TM, Bahn JH, Chew Jg, Xiao X, Schlinger BA (2016) Research resource:

373   Hormones, genes, and athleticism: Effect of androgens on the avian muscular transcriptome. Mol

374   Endocrinol 30:254-271. doi: 10.1210/me.2015-1270

375   Haines RW (1939). A revision of the extensor muscles of the forearm in tetrapods. J Anat 73:211-233.

376   Hale ME, Long JH Jr., McHenry MJ, Westneat MW (2002) Evolution of behavior and neural control of

377   the fast-start escape response. Evolution 56:993-1007

378   Herbst KL, Bhasin S (2004) Testosterone action on skeletal muscle. Curr Opin Clin Nutr Metab Care

379   7:271-277

380   Herrel A, Vanhooydonck B, Porck J, Irschick DJ (2008) Anatomical basis of differences in locomotor

381   behavior in *Anolis* lizards: A comparison between two ecomorphs. Bull Mus Comp Zool 159:

382   213-238. doi: 10.3099/0027-4100-159.4.213

383   Hertz PE, Huey RB, Stevenson RD (1993) Evaluating temperature regulation by field-active ectotherms –

384   the fallacy of the inappropriate question. Am Nat 142:796-818. doi: 10.1086/285573

385   Hicks RA, Trivers RL (1983) The social behavior of *Anolis valencienni*. In: Rhodin AGJ, Miyata K (eds)

386   Advances in Herpetology and Evolutionary Biology: Essays in Honor of Ernest E. Williams.

387   Museum of Comparative Zoology, Cambridge. pp 570-595

388   Holmes MM, Wade J (2005). Testosterone regulates androgen receptor immunoreactivity in the

389   copulatory, but not courtship, neuromuscular system in adult male green anoles. J

390   Neuroendocrinol 17(9):560-569. doi: 10.1111/j.1365-2826.2005.01339.x

391   Holmes MM, Bartrem CL, Wade J (2007) Androgen dependent seasonal changes in muscle fiber type in

392   the dewlap neuromuscular system of green anoles. Physiol Behav 91:601-608. doi:

393   10.1016/j.physbeh.2007.03.022

394   Husak JF, Lovern MB (2014) Variation in steroid hormone levels among Caribbean *Anolis* lizards:

395   Endocrine system convergence? Horm Behav 65(4):408-415. doi: 10.1016/j.yhbeh.2014.03.006

396 Husak JF, Irschick DJ, Meyers JJ, Lailvaux SP, Moore IT (2007) Hormones, sexual signals and

397 performance of green anole lizards (*Anolis carolinensis*). Horm Behav 52:360-367. doi:

398 10.1016/j.yhbeh.2007.05.014

399 Huyghe K, Herrel A, Vanhooydonck B, Meyers JJ, Irschick DJ (2007) Microhabitat use, diet, and

400 performance data on the Hispaniolan twig anole, *Anolis sheplani*: Pushing the boundaries of

401 morphospace. Zoology 110(1):2-8

402 Irschick DJ, Losos JB (1998) A comparative analysis of the ecological significance of maximal locomotor

403 performance in Caribbean *Anolis* lizards. Evolution 52:219-226. doi: 10.2307/2410937

404 Jenssen TA (1977) Evolution of anoline lizard display behavior. Amer Zool 17(1):203-215. doi:

405 10.1093/icb/17.1.203

406 Jenssen TA, Gladson NL (1984) A comparative display analysis of the *Anolis brevirostris* complex in

407 Haiti. J Herpetol 18:217-230

408 Jenssen TA, Nunez SC (1998) Spatial and breeding relationships of the lizard, *Anolis carolinensis*:

409 evidence of intrasexual selection. Behaviour 135:981-1003

410 Jenssen TA, Orrell KS, Lovern MB (2000) Sexual dimorphisms in aggressive signal structure and use by

411 a polygynous lizard, *Anolis carolinensis.* Copeia 2000(1):140-149. doi: 10.1643/0045-

412 8511(2000)2000[0140:SDIASS]2.0.CO;2

413 Johnson MA, Leal M, Schettino LR, Lara AC, Revell LJ, Losos JB (2008) A phylogenetic

414 perspective on foraging mode evolution and habitat use in West Indian *Anolis* lizards. Anim

415 Behav 75: 555-563. doi: 10.1016/j.anbehav.2007.06.012

416 Johnson MA, Wade J (2010) Behavioural display systems across nine *Anolis* lizard species: Sexual

417 dimorphisms in structure and function. Proc Roy Soc B 277(1688):1711-1719. doi:

418 10.1098/rspb.2009.2323

419 Johnson MA, Revell LJ, Losos JB (2010) Behavioral convergence and adaptive radiation: effects of

420 habitat use on territorial behavior in *Anolis* lizards. Evolution 64:1151-1159. doi: 10.1111/j.1558-

421 5646.2009.00881.x

422   Johnson MA, Lopez MV, Whittle TK, Kircher BK, Dill AK, Varghese D, Wade J (2014) The evolution

423   of copulation frequency and the mechanisms of reproduction in male *Anolis* lizards. Current

424   Zoology 60:768-777. doi: 10.1093/czoolo/60.6.768

425   Kamath A, Losos JB (2017) The erratic and contingent progression of research in territoriality: a case

426   study. Beh Ecol Sociobiol 71:89. doi: 10.1007/s00265-017-2319-z

427   Leal M, Rodriguez Robles JA (1997) Signaling displays during predator–prey interactions in a Puerto

428   Rican anole, *Anolis cristatellus*. Anim Behav 54:1147-1154. doi: 10.1006/anbe.1997.0572

429   Losos JB (1990) Concordant evolution of locomotor behavior, display rate, and morphology in West

430   Indian *Anolis* lizards. Anim Behav 39:879-890. doi: 10.1016/S0003-3472(05)80952-2

431   Losos JB (2009) Lizards in an Evolutionary Tree. University of California Press, Berkeley

432   Mangiamele LA, Fuxjager MJ, Schuppe ER, Taylor RS, Hödl W, Preininger D (2016) Increased

433   androgenic sensitivity in the hind limb musculature system marks the evolution of a derived

434   gestural display. Proc Natl Acad Sci USA 113:5664-5669. doi: 10.1073/pnas.1603329113

435   Neal JK, Wade J (2007) Androgen receptor expression and morphology of forebrain and neuromuscular

436   systems in male green anoles displaying individual differences in sexual behavior. Horm Behav

437   52(2):228-236. doi: 10.1016/j.yhbeh.2007.04.009

438   Nicholson KE, Harmon LJ, Losos JB (2007) Evolution of *Anolis* lizard dewlap diversity. PLoS ONE

439   2:e274. doi: 10.1371/journal.pone.0000274

440   Ord TJ, Martins EP (2006) Tracing the origins of signal diversity in anole lizards: phylogenetic

441   approaches to inferring the evolution of complex behaviour. Anim Behav 71:1411-1429. doi:

442   10.1016/j.anbehav.2005.12.003

443   Orell KS, Jenssen TA (1998) Display behavior of the lizard *Anolis bahorucoensis*: an anole with a

444   diminuitive dewlap. Carib J Sci 34:113-125

445   Peters SE, Aulner DA (2000) Sexual dimorphism in forelimb muscles of the bullfrog, *Rana catesbeiana*:

446   a functional analysis of isometric contractile properties. J Exp Biol 203:3639-3654.

447 Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2017) nlme: Linear and nonlinear mixed effects

448   models. R package version 3.1-131. R Foundation for Statistical Computing

449 Prum RO (1990) Phylogenetic analysis of the evolution of display behavior in the neotropical manakins

450   (Aves: Pipridae). Ethol 84:202-231.

451 Pyron RA, Burbrink FT, Wiens JJ (2013) A phylogeny and revised classification of Squamata, including

452   4161 species of lizards and snakes. BMC Evol Biol 13:93. doi: 10.1186/1471-2148-13-93

453 Queral A, Garcia R, Powell R, Parmerlee JS, Smith DD, Lathrop A (1995) Agonistic responses by a grass

454   anole, *Anolis olssoni* from the Dominican Republic, to male conspecifics. Amphibia-Reptilia

455   16:313-321. doi: 10.1163/156853895X00398

456 Rand AS (1967) Ecology and social organization in the iguanid lizard *Anolis lineatopus*. Proc US Natl

457   Mus 122:1-79. doi: 10.5479/si.00963801.122-3595.1

458 Rand MN, Breedlove SM (1992) Androgen locally regulates rat bulbocavernosus and levator ani size. J

459   Neurbiol 23:17-30

460 R Core Team (2014). R: A language and environment for statistical computing. R Foundation for

461   Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

462 Regnier M, Herrera AA (1993) Changes in the contractile properties by androgen hormones in sexually

463   dimorphic muscles of male frogs (*Xenopus laevis*). J Physiol 461:565-581

464 Rubinstein NA, Erulkar SD, Schneider GT (1983) Sexual dimorphism in the fibers of a 'clasp' muscle of

465   *Xenopus laevis*. Exp Neurol 82:424-431

466 Sanger TJ, Seav SM, Tokita M, Langerhans RB, Ross LM, Losos JB, Abzhanov A (2014) The oestrogen

467   pathway underlies the evolution of exaggerated male cranial shapes in *Anolis* lizards. Proc Roy

468   Soc B 281:20140329. doi: 10.1098/rspb.2014.0329

469 Stamps JA (1977) Social behavior and spacing patterns in lizards. In: Gans C, Tinkle DW (eds) Biology

470   of the Reptilia, Vol. 7. Academic Press, London. pp 265-334

471 Stamps J (1994). Territorial behavior: Testing the assumptions. Adv Study Behav. 23:173-232

472   Suzuki D, Murakami G, Minoura N (2002) Histology of the bone-tendon interfaces of limb muscles in

473   lizards. Ann Anat 184:363-377. doi: 10.1016/S0940-9602(02)80057-7

474   Tokarz RR, McMann S, Smith LC, John-Alder H. (2002) Effects of testosterone and season on the

475   frequency of dewlap extensions during male–male interactions in the lizard *Anolis sagrei*. Horm

476   Behav 41:70-79. doi: 10.1006/hbeh.2001.1739

477   Veney SL, Wade J (2004) Steroid receptors in the adult zebra finch syrinx: A sex difference in androgen

478   receptor mRNA, minimal expression of estrogen receptor alpha and aromatase. Gen Comp

479   Endocrinol 136:192-199. doi: 10.1016/j.ygcen.2003.12.017

480   Wade J (2012) Sculpting reproductive circuits: Relationships among hormones, morphology and behavior

481   in anole lizards. Gen Comp Endocrinol 176:456-460. doi: 10.1016/j.ygcen.2011.12.011

482   Zheng Z, Cohn MJ (2011) Developmental basis of sexually dimorphic digit ratios. Proc Natl Acad Sci

483   USA 108:16289-16294. doi: 10.1073/pnas.1108312108

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Figure legends

Figure 1. Phylogeny of the six *Anolis* lizard species included in this study, pruned from the squamate

phylogeny of Pyron et al. (2013). Photograph of *A. bahorucoensis* by Thomas J. Sanger.

Figure 2. Androgen receptor (AR) expression in biceps muscles and a) rate of locomotor movements and

b) rate of social pushup displays in six *Anolis* species.

Figure 3. Circulating testosterone and androgen receptor (AR) expression in bicep muscles of four *Anolis*
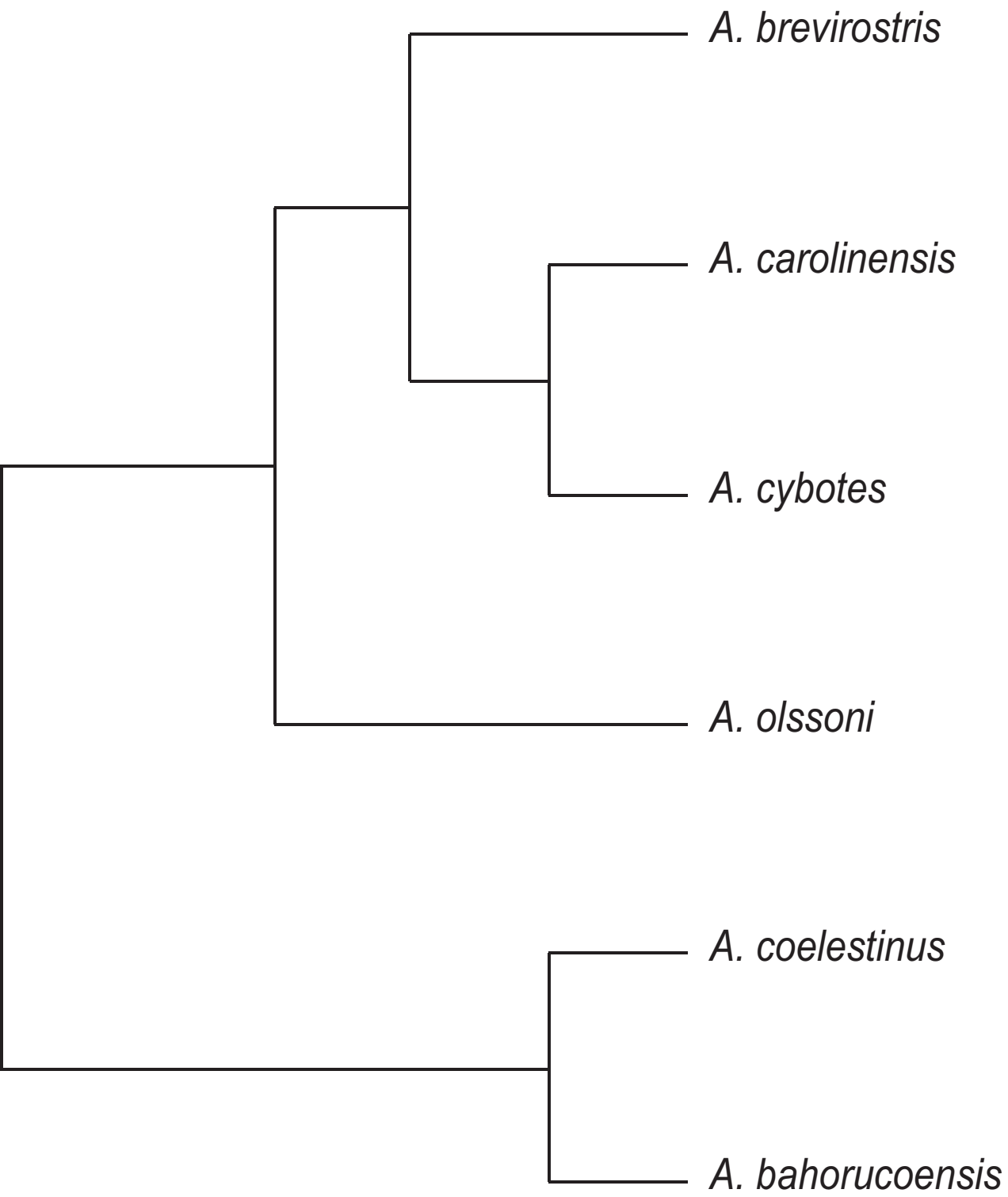
lizard species.

Note: All figures were created in Adobe Illustrator.

496   **Table 1.** Localities of behavioral observations of six *Anolis* lizard species.

Species	Number observed	Hours observed	Year(s) of observation	Locality	GPS coordinates
A. bahorucoensis	10	23.2	2006	Polo, Dominican Republic	18.105, -71.276
A. brevirostris	85	57.5	2011, 2015	Coralsol Beach Resort, Barahona, Dominican Republic	18.062, -71.111
A. carolinensis	107	123.3	2010, 2014	Palmetto State Park, Gonzales, Texas, USA	29.593, -97.585
A. coelestinus	87	60.0	2006, 2011, 2015	Coralsol Beach Resort, Barahona, Dominican Republic	18.062, -71.111
A. cybotes	113	74.1	2006, 2011, 2015	Coralsol Beach Resort, Barahona, Dominican Republic	18.062, -71.111
A. olssoni	31	49.6	2006, 2015	Coralsol Beach Resort, Barahona; and Bani, Dominican Republic	18.062, -71.111; 18.232, -70.347

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498

499 **Table 2.** Average (SE) of male body size, behavior, bicep fiber size, and the proportion of AR+ nuclei in

500 bicep muscles of six *Anolis* lizard species. Superscripts indicate results of Tukey's HSD post-hoc tests

501 following ANOVA comparing across species; species with different superscripts for a trait were

502 significantly different from one another.

Species	Mass (g)	Push-ups/min	Total move-ments/min	Runs/ min	Crawls/ min	Jumps/ min	Bicep fiber size (µm²)	Proportion AR+ nuclei
A. bahorucoensis	1.30 (0.08)	0.050[a] (0.033)	0.209[a] (0.069)	0.006[a] (0.002)	0.126[a] (0.042)	0.077[a] (0.022)	- -	0.305[a] (0.040)
A. brevirostris	2.60 (0.15)	0.859[bc] (0.103)	0.669[b] (0.078)	0.177[bc] (0.039)	0.442[b] (0.062)	0.050[a] (0.009)	2912 (169)	0.295[a] (0.031)
A. carolinensis	5.53 (0.25)	8.862[d] (0.697)	1.820[c] (0.119)	0.444[c] (0.058)	1.103[c] (0.058)	0.273[b] (0.021)	2166 (116)	0.385[a] (0.052)
A. coelestinus	6.16 (0.37)	1.609[bc] (0.235)	0.730[b] (0.069)	0.093[ab] (0.016)	0.520[b] (0.042)	0.117[a] (0.022)	3422 (165)	0.284[a] (0.022)
A. cybotes	7.95 (0.42)	2.201[c] (0.262)	0.388[a] (0.027)	0.132[ab] (0.017)	0.165[a] (0.015)	0.091[a] (0.011)	3896 (342)	0.255[a] (0.024)
A. olssoni	1.24 (0.07)	0.384[ab] (0.114)	0.321[a] (0.073)	0.051[ab] (0.022)	0.145[a] (0.030)	0.125[a] (0.025)	1586 (81)	0.298[a] (0.059)

503

504

Figure 1

A. brevirostris

A. carolinensis

A. cybotes

A. olssoni

A. coelestinus

A. bahorucoensis

Figure 2

a

b

Figure 3